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Imaging of Tau and Microtubules to Study Mechanisms of Tau Pathologies and Neurodegeneration

Faculty of Biology and Chemistry

Osnabrück University

Presented by

Christian Conze

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CONTENTS

ABSTRACT

The nervous system undergoes constant remodeling and adapting of its structural organization to fulfill its function, the propagation and processing of information. The structural backbone of neurons are arrays of microtubules (MTs) maintaining their specialized morphologies and serving as railway system for the transport of cargoes and information. The functional regulation of this complex system is based on many different factors, while microtubule-associated proteins (MAPs) like tau being one of them. The tau protein is of particular interest as it plays a central role in numerous neurodegenerative diseases, called tauopathies, where Alzheimer's Disease (AD) is the most common and prominent example. Hallmark of tauopathies such as AD is the aggregation and increased phosphorylation of tau, while also many other post-translational modifications (PTMs) of the protein are described and linked to neurodegeneration.

Scope of this thesis are investigations on how the neuronal MTs, MT-dynamics and MT-dependent transport are affected by post-translational changes of the tau protein. A new type of toxic gain of function of tau is described, as it impairs axonal transport when cleaved at a diseases relevant site and microtubule-targeting drugs are introduced as pharmacological modifiers of MAP-microtubule interaction. The effect of MT-stabilization by Epothilone D on the MT-cytoskeleton as well as the effect of a tau knockout is further investigated by cutting-edge microscopy (DNA-PAINT SMLM) and algorithm-based quantification of the MT array. Changes of the MT organization and structure on the level of individual MTs upon stabilization and tau knockout are described. Accumulating evidence suggests that pathologically modified monomeric and soluble oligomeric forms of tau should be considered as harmful tau species. Hence, a cell-based model allowing investigations of disease-like modified tau, its oligomerization, and MT-interaction is implemented enabling the screening for modulators of early-stage aggregation and phosphorylation of tau. Potential drug candidates with polypharmacological activity are presented.

INTRODUCTION

Neuronal Microtubules

The human brain is thought to contain more than 85 billion neurons (Azevedo et al., 2009; Bartheld et al., 2016). The function of neurons is the propagation and processing of information provided by electrical or chemical signals. This requires a complex structural organization and dynamic remodeling of the nervous system. Along with the actin cytoskeleton, the microtubule (MT) cytoskeleton is fundamental for cell proliferation and the major developmental changes as nerve cells migrate, differentiate, develop dendrites and axons and establish synaptic connections with other neurons (Kapitein and Hoogenraad, 2015). Neurons are highly polarized cells with single long axons extending from the cell body, transmitting signals, and multiple shorter dendrites receiving signals. MTs are aligned into longitudinal parallel bundles that run along axons and dendrites, often accompanied by neuronal intermediate filaments (neurofilaments). Neurofilaments are known to regulate the diameter of axons and are especially abundant in large-caliber axons of vertebrates (Prokop, 2020). However, the structural backbone of neurons is the array of MTs maintaining their specialized morphologies. In addition, they serve as long-distance railways for proteins, organelles, and themselves to be actively transported in both directions within axons and dendrites. Vertebrates express multiple α- and β-tubulin genes, and *in vitro* α- and β-tubulin heterodimers can nucleate *de novo* and make up MTs at a certain concentration of tubulin and in presence of GTP with ambient buffer and temperature conditions. Subsequent to MT assembly, the GTPase activity of β-tubulin hydrolyzes GTP to guanosine diphosphate (GDP) and it becomes non-exchangeable when build into the tubulin polymer (David-Pfeuty et al., 1977). The heterodimers bind in a head-to-tail fashion into protofilaments which associate into hollow cylindrical structures of about 25 nm in diameter. Their slow-growing minus-end exposes α-tubulin and the fast-growing plus-end β-tubulin. In living cells, the *de novo* nucleation is suppressed. Here, MTs of mostly 13 protofilaments nucleate from microtubule organization centers in the soma containing γ-tubulin ring complexes (Tilney et al., 1973; Oakley et al., 2015). γ-tubulin binds specifically to α -tubulin of the α-β-heterodimer establishing the polar orientation of MTs. In non-neuronal cells microtubules stay attached to the nucleating structures, while MTs of neurons can be released, captured by other proteins, and transported away by molecular motor proteins. These molecular motors move MTs specifically with their plus-end leading into axons and dendrites or additionally with the minus-end leading into dendrites (Baas et al., 2016). Nevertheless, it was also reported that local nucleation of MTs may occur in axons

and dendrites during neuronal maturation (Stiess et al., 2010). The MT-array extends from the soma to the tips of axons and dendrites but individual MTs do not span the entire length. While some MTs are less than a micrometer in length, others can extend to 100 µm or even more. The dimensions of neuronal MT length varies depending on organisms and cell types but at least in developing axons and dendrites of mammals, average MTs are just several micrometers long (Weiss and Mayr, 1971; Chalfie and Thomson, 1979; Bray and Bunge, 1981; Letourneau, 1982; Joshi et al., 1986; Okabe and Hirokawa, 1988; Yu and Baas, 1994; Yogev et al., 2016; Nishida et al., 2020). However, most MTs do not have a steady length anyhow. *In vitro* as well as *in vivo* MTs undergo rapid assembly and disassembly governed by a mechanism termed dynamic instability (Mitchison and Kirschner, 1984). The mechanism is depending on the hydrolysis of GTP-tubulin which occurs principally at the plus-end. If the GTP-hydrolysis catches up with the addition of new tubulin heterodimers, the MT exhibits a fast disassembly. Hence, if a GTP-cap is present at the plus-end, the MT can keep assembling (**Figure 1**).

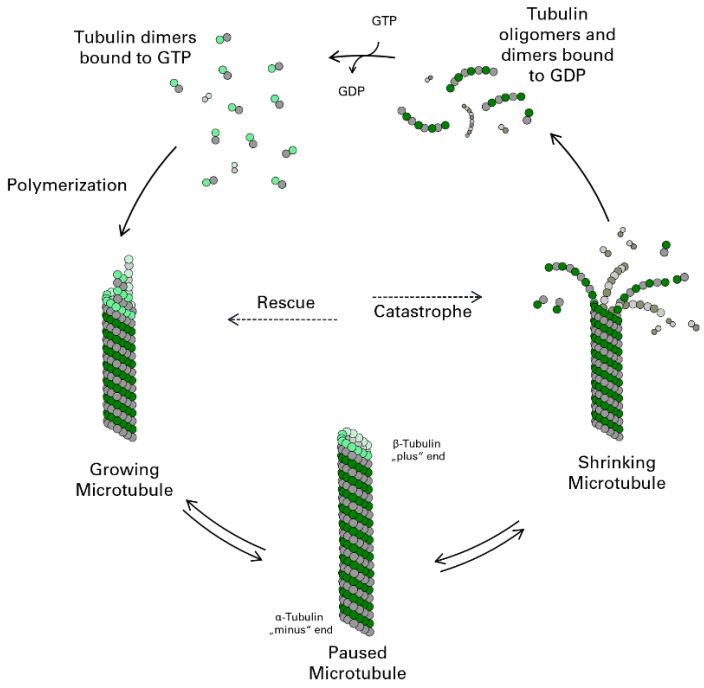

Figure 1 Dynamic instability model of MTs. A single MT consist usually of 13 protofilaments that associate laterally to hollow cylinders of about 25 nm in diameter. The head-to-tail association of the αβ-heterodimers makes MTs polar structures, with different polymerization rates at the two ends. In each protofilament, the αβ-heterodimers are oriented with their β -tubulin monomer pointing towards the faster-growing (plus) end and their α-tubulin monomer exposed at the slower-growing (minus) end. The plus-end generally has a GTP cap of at least one tubulin layer that stabilizes the MT structure. When this GTP cap is lost, the protofilaments splay apart and the MT depolymerizes. During or shortly after polymerization, the tubulin subunits hydrolyze their bound GTP and become non-exchangeable. Thus, the MTs are mainly composed of GDP–tubulin, while depolymerization is characterized by the rapid loss of GDP–tubulin subunits and oligomers from the MT plus-end. The transition between growth and shrinkage is also termed rescue and catastrophe.

Microtubule Regulating Factors

Another feature of the dynamic instability model is called selective stabilization (Kirschner and Mitchison, 1986). By visualizing MT plus-ends it was shown that MTs remain highly dynamic and are labile structures in axons as well in dendrites (Stepanova et al., 2003; Kleele et al., 2014). Nevertheless, neurons display also stable and long-lived MTs that are acetylated and detyrosinated. These stable MTs still show dynamic behavior, while a subpopulation of long-lived MTs is hyperstable and do not exhibit any dynamic tubulin turnover. These hyperstable MTs are in addition polyaminated and resistant to cold, calcium, or MT-depolymerizing drugs (Matamoros and Baas, 2016). Polyamination was shown to actually stabilize MTs (Song et al., 2013) and is thereby different from acetylation and detyrosination which accumulate on long-lived MTs but do not confer stability (Howes et al., 2014; Janke and Magiera, 2020). It was assumed for long that proteins binding to the MT lattice confer stability, but it remains poorly understood which and how such microtubule-associated proteins (MAPs) exhibit this functionality. In neurons, typical MTs of axons and dendrites consist of stable and labile domains, with the stable domain towards the minus-end and the labile part at the plus-end. Concerning the overall MT mass, a higher percentage of MTs is stable in the axon compared to dendrites (Baas et al., 2016) (**Figure 2**).

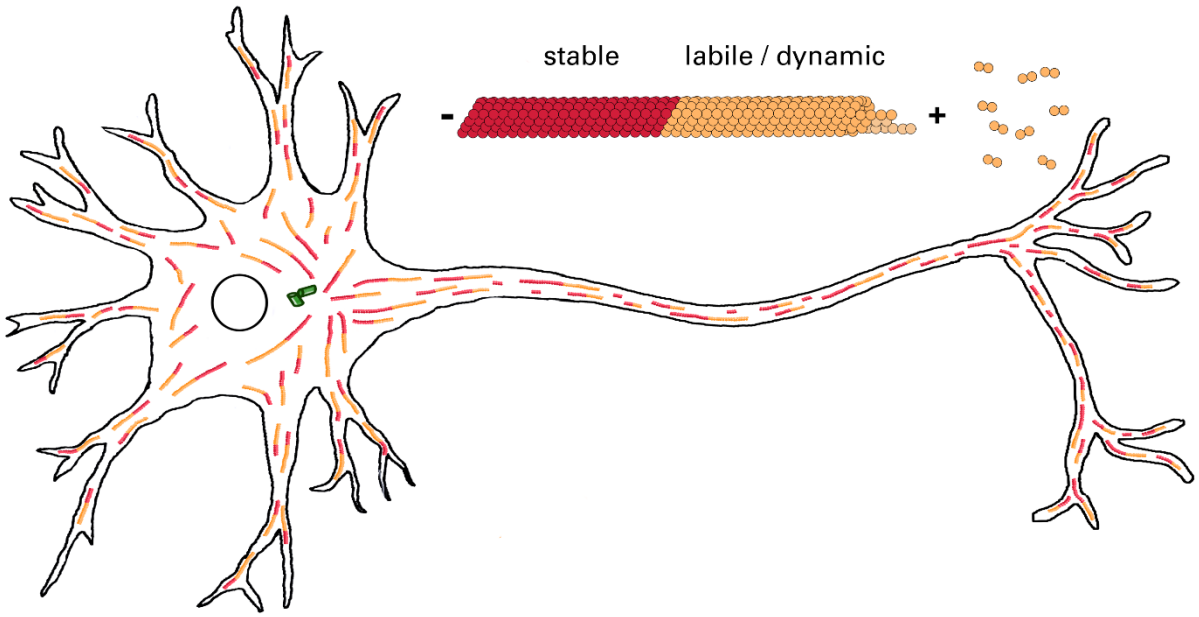

Figure 2 Axonal and dendritic organization of MTs in vertebrate neurons. The schematic depicts a typical neuron with one axon and multiple dendrites. MTs are uniformly orientated in the axonal compartment while having a mixed orientation in dendrites. In the axon as well as in dendrites, MTs consist of stable (red) and labile (orange) domains. In the axon, a fraction of short MTs is hyperstable and an overall higher percentage of the total MT mass is stable compared to dendrites, where MTs are more dynamic. Neither axonal nor dendritic MTs are attached to the centrosome (green).

However, the stability of MTs varies along the axon and the polymer turnover is more rapid in its distal part (Brown et al., 1993; Ahmad et al., 1993). MAPs that are thought to be involved

in axonal MT stabilization such as tau or MAP1b are more enriched in the distal region of axons and on labile domains of MTs, suggesting that these proteins are important for regulating assembly rather than conferring stability (Black et al., 1994; Black et al., 1996; Qiang et al., 2018). Overexpression of tau or MAP2, which belong to the same family of MAPs, does not affect the rates of MT growth or shrinkage (Kaech et al., 1996). On the other hand, the depletion of tau in primary rat neurons results in less dynamic MTs and a loss of the liable MT mass. MAP6 in contrast is enriched on stable domains of MTs (therefore also called STOP, stable tubulin only polypeptide). When tau is depleted from cells, MAP6 shows a broader distribution across the MTs and an increase in stable MT mass is seen, which can however not fully compensate for the net loss in MT mass caused by tau depletion (Qiang et al., 2018). A depletion of MAP6 in turn leads to an increase in dynamic MTs, accompanied by a decrease in the stable MT mass, and makes MAP6 therefore a better candidate to be an actual stabilizer of MTs (Tortosa et al., 2017). Anyways, MTs can be stabilized by capturing their plus-ends by plus-end tracking proteins (+TIPs) that promote MT polymerization. The +TIPs include autonomous binding ones such as the XMAP215 family members of microtubule polymerases and the end binding proteins EB1, EB2, and EB3 (Akhmanova and Steinmetz, 2015). Other +TIPs such as CLIPs or CLAPS can in turn be recruited by the autonomous binding tip trackers and can enable linkage to structures of the cell cortex (Lansbergen et al., 2006). There is actually not too much known about the interplay between classical MAPs such as tau, MAP2, or MAP1b with +TIPs but it was shown that those MAPs can control EB1 and EB3 localization and function in developing neurons of rodents (Kapitein et al., 2011; Tortosa et al., 2013; Sayas et al., 2015; Hahn et al., 2021) While *in vitro* the addition of tubulin, albeit slower, can also occur at the minus-end, *in vivo* the minus-ends appear to be completely non-dynamic. In living cells, minus-end targeting proteins (-TIPs) such as CAMSAPs (calmodulin-regulated spectrin associated proteins) can bind free minus-ends and block the addition of heterodimers from the tubulin pool in various cell types (Hendershott and Vale, 2014; Akhmanova and Hoogenraad, 2015). CAMSAPs were shown to be required for neuronal polarity, axon specification, and dendritic branch formation (Yau et al., 2014). Immunostaining of CAMSAPs reveals stretches along the MT minus-end (Jiang et al., 2014), and it was shown that these can only occur when γ-tubulin ring complexes are detached from MT minus-ends or when a minus-end is created by MT cutting or breakage (Yau et al., 2014). Proteins that cut or break MTs, so-called MT-severing proteins are AAA-Enzymes family members (ATPases Associated with diverse cellular Activities). Three MT-severing proteins are known so far, namely katanin, spastin, and fidgetin (McNally and Roll-Mecak, 2018). MT severing at the minus-end of MTs enables the release from the centrosome so that MTs can be transported to the axonal or dendritic

compartment of the cell (Baas et al., 2016). Katanin as well as spastin have a preference for acting on MTs at stable domains which are post-translationally modified by acetylation or polyglutamylation (Sudo and Baas, 2010; Lacroix et al., 2010) while fidgetin shows a preference for non-acetylated MT segments of the labile domains (Baas and Ahmad, 2013) (**Figure 3**).

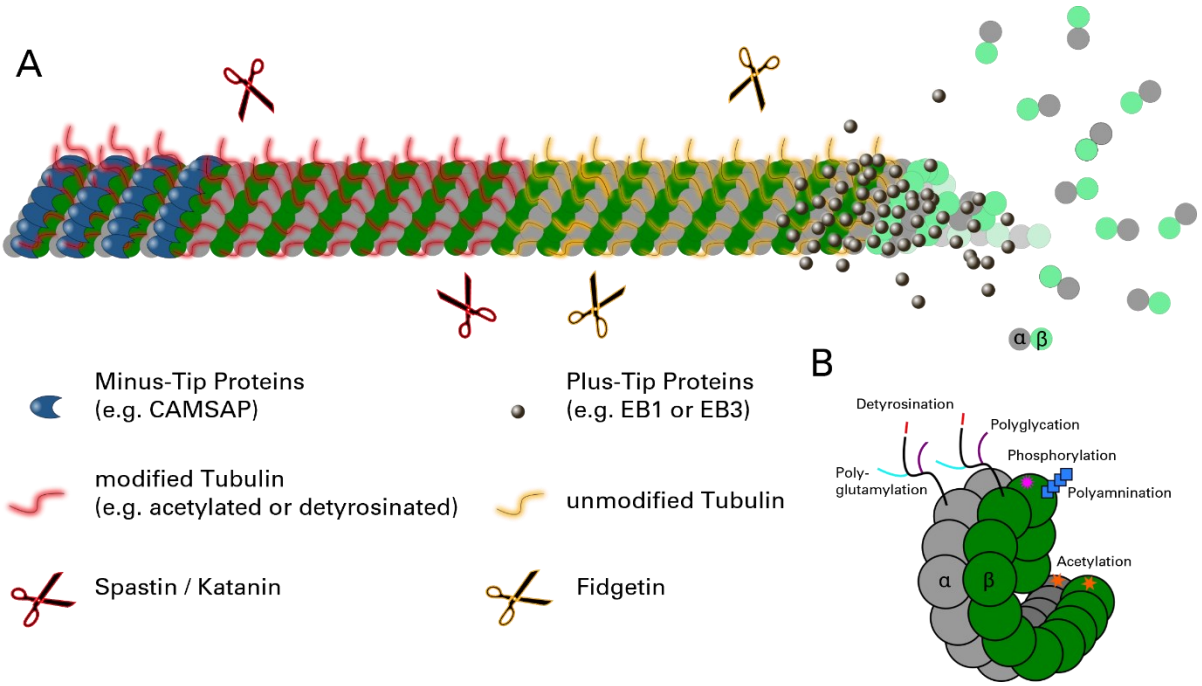

Figure 3 Stability domains of neuronal MTs with related molecules and post-translational modifications. **A** Neuronal MTs have stable domains towards their minus-end, that are post-translationally modified by acetylation or detyrosination for example. At the minus-end, minus-tip proteins accumulate, which prevent MT disassembly. Towards the plus-end, MTs are labile and assemble and disassemble dynamically. The plus-tip dynamics are associated with dynamic interactions with plus-tip proteins that promote MT polymerization. Both stability domains differ in their interaction with various MT related and associated proteins. The MT-severing proteins katanin and spastin target stable domains, while fidgetin targets the labile domains. **B** More detailed schematic of tubulin PTMs and where they occur. Ploygluta mylation (light blue), Polyglycation (violet), and Detyrosination (red) take place on the C-terminal "tails" of tubulin dimers. Phosphorylation (pink asterisk) and Polyamination (blue squares) appear on residues of β-tubulin, while acetylation (orange asterisk) takes place on the luminal side of the MTs on both subunits of the αβ-heterodimers.

Beyond fidgetin, other proteins are depolymerizing MTs from the plus-end. Stathmins shift MTs towards disassembly by sequestering heterodimers and hinder thereby polymerization. Furthermore, kinesin superfamily proteins (KIFs), originally identified as molecular motors for axonal transport (Vale et al., 1985), have shown to have MT stabilizing as well as depolymerizing activities. Members of the Kinesin-4 and -11 family were shown to suppress MT dynamics and bundle MTs in neurons and other cell types (Niwa, 2015). Members of the Kinesin-13 family, such as mitotic centromere-associated kinesin (MCAK) can depolymerize MTs from the plus- and minus-end (Desai et al., 1999). Those kinesins are non-motile but like the motor activity of other KIFs, MT depolymerization is depending on

ATP breakdown. Non-motile kinesins are thought to diffuse along the MTs and dissemble MTs by promoting a curved conformation of protein dimers and MT ends. (Moores et al., 2002; Asenjo et al., 2013).

In addition, several other factors are known to contribute to MT regulation, especially in terms of MT destabilization, and play a role in axon degeneration, axonal loss, and dendritic simplification as features of neurodegenerative diseases (Brandt and Bakota, 2017). Region-specific dendritic simplification induced by amyloid-beta (Aβ) through NMDA receptor activation, is associated with MT-destabilization and can be rescued by receptor inhibition and treatment with MT-stabilizing agents (Golovyashkina et al., 2015; Penazzi et al., 2016). In other studies, it could be shown that pathogenic insults, such as excitotoxicity, induce axon degeneration which can be inhibited by MT-stabilizing agents (King et al., 2013). Key effect in excitotoxic insults is an NMDA and AMPA receptor-mediated intracellular calcium (Ca^{2+}) increase (Arundine and Tymianski, 2003; Szydlowska and Tymianski, 2010). In fact, a link between elevated Ca^{2+} levels and MT damage in non-neuronal cell types as well as neurons has been known for long (Weisenberg, 1972; O'Brien et al., 1997). However, most glutamate receptors are localized on the somatodendritic compartment and thereby spatially separated from the axon. Within the axon extracellular influx through voltage-gated Ca^{2+} channels, transient receptor potential channels or reverse operation of sodium/calcium exchanger are possible but the axon contains also its own calcium stores (Szydlowska and Tymianski, 2010). The endoplasmic reticulum (ER), which extends into the axon, is an important storage of intracellular Ca^{2+} and the ER is tightly coupled to mitochondria which are another important source of intracellular Ca^{2+} (Wu et al., 2017; Luarte et al., 2018). By live-cell imaging of primary mouse cortical neurons, axonal degeneration was seen upon excitotoxic kainic acid stimulation of the somatodendritic compartment which induced Ca^{2+} release of the axonal ER and mitochondria. A treatment with MT-stabilizing drugs prevented axonal fragmentation. A reduced axonal Ca^{2+} release was shown, an increase in acetylated and decrease in tyrosinated MTs was seen, suggestive of a more stable MT pool in the axon (Tian et al., 2020). Furthermore, a study from the early 90s using PC12 cells suggested that reactive oxygen species (ROS) mediate their destructive effect on MTs via elevation of intracellular Ca^{2+} (Hinshaw et al., 1993). More recent evidence suggests that a tightly controlled redox balance regulates MT dynamics in physiological as well as pathological contexts. While physiological ROS production is needed for a proper cytoskeletal organization in neurons, oxidation tends to disrupt polymerization and interferes with cytoskeletal dynamics (Wilson and Gonzalez-Billault, 2015). The regulation of MT dynamics by ROS and ROS-signaling is thought to be affected by several factors

including Ca^{2+} homeostasis as well as PTMs of tubulin via cysteine residues (Sparaco et al., 2006). The redox state of these residues is linked to GTP binding of tubulin and MT polymerization, and high ROS levels lead to MT depolymerization (Luduena and Roach, 1991; Landino et al., 2004a). Another factor involved in ROS-mediated MT regulation might be MAPs such as tau or MAP2. Both of them contain several cysteine residues, and oxidation of those residues decreases MT polymerization *in vitro* suggesting an indirect involvement of the redox balance on MT dynamics (Landino et al., 2004b). The inhibition of ROS below physiological levels and its effects on MT dynamics has not been explored so far. However, there is good evidence that changes in redox- or Ca^{2+} homeostasis contribute to the regulation of MT dynamics and have impacts on MT-dependent processes such as transport.

Microtubule Dependent Transport

Apart from its function as structural entity enabling and preserving the specified morphologies and plasticity of neurons, the MT cytoskeleton is the key player in the propagation and processing of information in view of transport processes. Intracellular transport is fundamental to function, developmental stages, plasticity, or survival of nerve cells in general and is almost entirely dependent on MTs. The interplay of molecular motors, adaptors and scaffolding proteins contributing to the transport machinery of individual cargos is organelle specific. The composition of this transport machinery determines different patterns of motility and the localization of the respective cargos (Maday et al., 2014). Early studies using live-cell imaging could visualize organelle transport for the first time in squid giant axons (Brady et al., 1982; Allen et al., 1982). A few years later the molecular motors kinesin (Vale et al., 1985) and dynein (Paschal et al., 1987) were discovered. In subsequent studies, kinesins and especially kinesin-1 were identified as major motor for transport towards the MT plus-end, which is the anterograde direction in axons, and dynein as motor for transport towards the minus-end, which is the retrograde direction in axons (Hirokawa et al., 1990; Hirokawa et al., 1991). Kinesin-1 family members are driving a wide range of cargos in neurons including vesicles, mitochondria, proteins, or RNA particles at velocities of up to ~ 0.5 to 1 µm/s. Kinesin-1 motors consist of a dimer of kinesin heavy chains and often but not always a dimer of kinesin light chains that contribute to the motor complex (Hirokawa et al., 2010). Dynein motors consist always of two heavy chains. This dimer is associated with intermediate and light chains that are binding to the tails of the heavy chain dimer forming the cargo binding domain. In addition, dynein depends on the dynein activator dynactin, a highly conserved multiprotein complex. Dynein motor complexes convey retrograde directed cargoes in axons and dendrites, such as brain-

derived neurotrophic factor (BDNF) vesicles, mitochondria, or endosomes at roughly the same velocities as kinesin-1 of up to ~ 0.5 to 1 µm/s (Maday et al., 2014) (**Figure 4**).

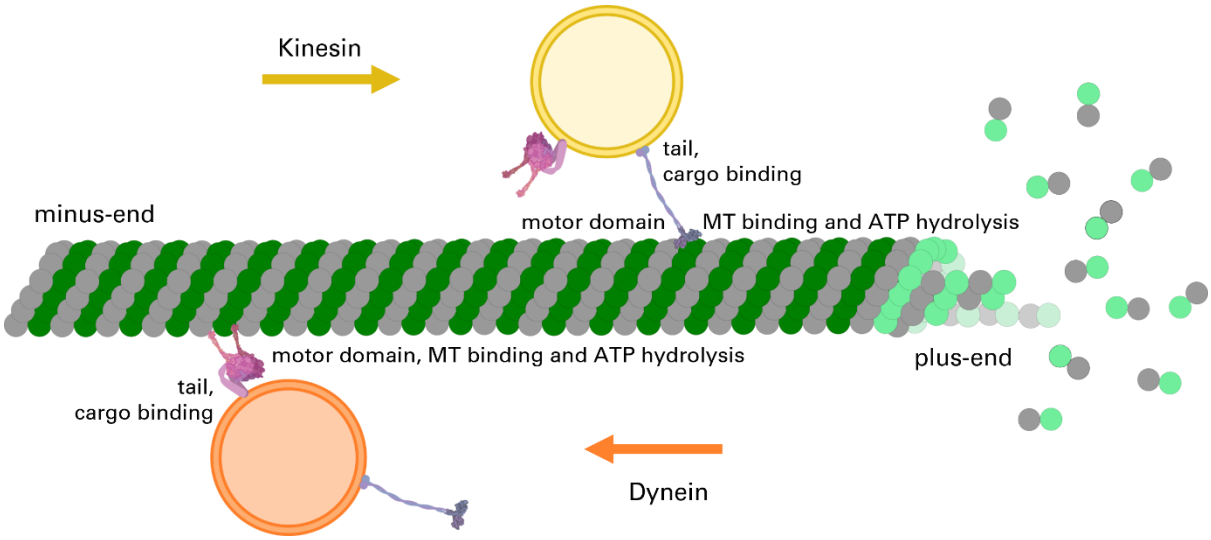

Figure 4 MT-based transport machinery. Schematic representation showing MT plus-end (kinesins, blue-grey) and minus-end (dynein, pink) directed molecular motors. The molecular motors bind to MTs via their globular head domains and hydrolyze ATP during movement. Cargos (yellow and orange) are bound to the tail domains of the motors either directly, through intermediate and light chains, or via adaptors.

The exact mechanisms of how transport is orchestrated are still not fully understood. Many cargoes have multiple motors bound simultaneously that may interact cooperatively or competitively resulting in the respective net motility of transport. The simplest model of transport suggests an unregulated tug-of-war between kinesin and dynein motors. Other models suggest a coordinated regulation of motors so that only one motor is active at a given time point. Intermediate models of coordinated transport suggest that one motor, e.g. kinesin, might be tightly regulated while the activity of the other motor is less controlled and is thereby overpowered although both may be active (Maday et al., 2014). However, there is growing evidence for the models of coordinated regulated transport mediated for example by PTMs of MT tracks, such as the tyrosination status (Janke and Magiera, 2020) or by polyglutamylation (Bodakuntla et al., 2020). Furthermore, it was shown *in vitro* that structural MAPs, such as tau, can influence and interfere with MT-dependent transport (Vershinin et al., 2007; Dixit et al., 2008). *In vitro* observations of localized dynamic condensations of tau suggest that those tau islands may act as roadblocks on MT tracks (Tan et al., 2019; Siahaan et al., 2019). Moreover, MAPs, such as MAP7, were shown to promote kinesin-based transport by recruiting kinesin-1 to MTs (Monroy et al., 2018). Other MAPs, such as +TIPs EB1 and -3 are capable of recruiting additional binding partners and many of them have a role in the localized regulation of transport processes (Moughamian et al., 2013). In addition, scaffolding proteins can act as binding platforms and mediate

molecular motor binding and action on MT tracks. Mitochondrial scaffolding proteins, such as Trafficking Kinesin Protein (TRAK), mediate motor protein binding with the mitochondrial membrane. TRAK can associate with kinesin-1 and dynactin and potentially steers bidirectional transport of mitochondria in axons and dendrites (van Spronsen et al., 2013). At neuronal synapses, which have a high energy demand, elevated Ca^{2+} levels are setting the association of mitochondrial scaffolding proteins with kinesins free leading to local sequestration of mitochondria (Fu and Holzbaur, 2014). Synapse-directed vesicles, such as amyloid precursor protein (APP) vesicles, bind the scaffolding protein JNK-interacting protein 1 (JIP1). The kinesin-1 driven transport of APP depends on the autoinhibition of the motor by binding the kinesin tail to its motor domain. This inhibition is relieved by the binding of JIP1 to the kinesin heavy chains. Binding of dynactin to JIP1 competitively inhibits the kinesin heavy chain and disrupts amyloid precursor protein (APP) transport. The affinity of JIP1 for kinesin1 or dynactin is controlled by JNK-dependent phosphorylation, acting as a molecular switch controlling the directionality of APP transport in neurons. Phosphorylation of JIP1 favors anterograde transport, while dephosphorylation gives preference to retrograde transport of APP vesicles (Fu and Holzbaur, 2013).

In addition, the local organization of the cytoskeleton can influence transport dynamics. The axon initial segment (AIS) for example exhibits MTs where the +TIPs EB1 and -3 interact with ankyrinG. Those MTs show very low dynamic behavior and can be seen as stabilized. Kinesins have been shown to recognize the entrance to the axon by preferentially binding to stabilized MTs (Leterrier et al., 2011). Axonal cargoes can enter the axon while dendritic ones arrest at the AIS. PTMs of MTs are thought to contribute to the regulation and sorting of axonal or dendritic cargos (Janke and Magiera, 2020). On the other hand, it has been proposed that the mixed microtubule polarity of dendrites may be sufficient for dynein motors to selectively enter the dendritic compartment (Kapitein et al., 2010). Other models propose an involvement of a dense actin cytoskeleton at the AIS as the key to filtering and sorting axonal and dendritic proteins and cargos (Watanabe et al., 2012; Huang and Rasband, 2016). An interplay between MT-dependent transport and the actin cytoskeleton is known from secretory and endocytic pathways where vesicular cargos are transferred between actin- and MT-tracks. The transport processes aren't as smooth as they may sound rather than being impeded by the cytoskeletal structures themself due to the high amount of intersecting filaments of different types and various motor binding of myosin, kinesin, and dynein motors (Ross et al., 2008a). As already mentioned, almost all cargos are simultaneously bound to anterograde and retrograde directed motors so that intracellular transport is achieved through a back-and-forth motion with an overall net directionality

towards the respective destination within the cell. It has been hypothesized that this seemingly inefficient transport behavior is actually a consequence to facilitate the maneuvering around roadblocks such as organelles, MAPs, or intersecting cytoskeletal tracks (Hancock, 2014). The effect of motor function and artificial cargos at MT-MT intersections was studied *in vitro* using reconstituted MTs on top of each other. Single motors and their density on cargoes revealed a varied behavior in terms of passing through, switching tracks, or dissociation at MT intersections (Ross et al., 2008b). In contrast to the highly processive kinesin motors, dynein motors perform more frequent back and side steps while moving along the MTs. On the other hand, kinesin-1 motors are more likely to detach from MTs compared to dynein when encountering obstacles on the MT tracks (Mallik et al., 2013). In non-neuronal cells, bidirectional lysosomal cargo transport was investigated and quantified using a correlative imaging method combining single-particle tracking with super-resolution microscopy of the MT cytoskeleton. Most lysosomes slowed down and stalled encountering an intersecting MT. The transport patterns correlated with the spatial separation of intersecting MTs and encountering them leads to long pauses in transport. However, the obstructions could be overcome with high fidelity by either switching tracks or passing through the intersection. Reversing directionality and moving backward on the same MT was a rather rare event. Retrograde cargos were more likely to pass through intersections than to switch tracks or reverse direction but pausing was seen in both directions with equal probability. The authors proposed a mechanistic model suggesting that MT-MT intersections larger than 100 nm are a minimal hindrance for forward motion and transport continues. A spatial separation less than ~100 nm of an intersecting MT represents a major obstacle stalling the motors and stopping forward motion until the obstacle can be overcome (Bálint et al., 2013). Furthermore, given that the distance that axonal and dendritic cargos are traveling is far longer than MT length, it is unlikely that transport takes place on a single MT. Consequently, cargos must switch MT tracks at their ends to reach their destination. In fact, according to mathematical modeling, the number and density of MTs within neurites are more important for efficient transport than individual MT length (Wortman et al., 2014). The low MT abundance in motor neurons of *C. elegans* allowed the investigations of MT lengths, number, and distance between adjacent MTs as well as a correlation with axonal transport using diffraction-limited light microscopy. It was shown that synaptic vesicle precursors pause at MT termini and that cargo pause time was inversely correlated with the local abundance of polymers. This is suggesting that MT coverage and switching tracks might be rate-limiting for efficient transport (Yogev et al., 2016). In a follow-up study, it was shown that vesicle pause times at MT-ends are shorter for anterograde motion compared to retrograde transport. Moreover, a small but significant

increase in reversals of directionality was seen at MT-ends compared to overall reversals upon individual tracks (Gramlich et al., 2021). These observation can also give an explanation for why peripheral axon branches of DRG neurons, which have more MTs than the central branch of the same neuron, display higher transport rates (Nascimento et al., 2018) (**Figure *5***).

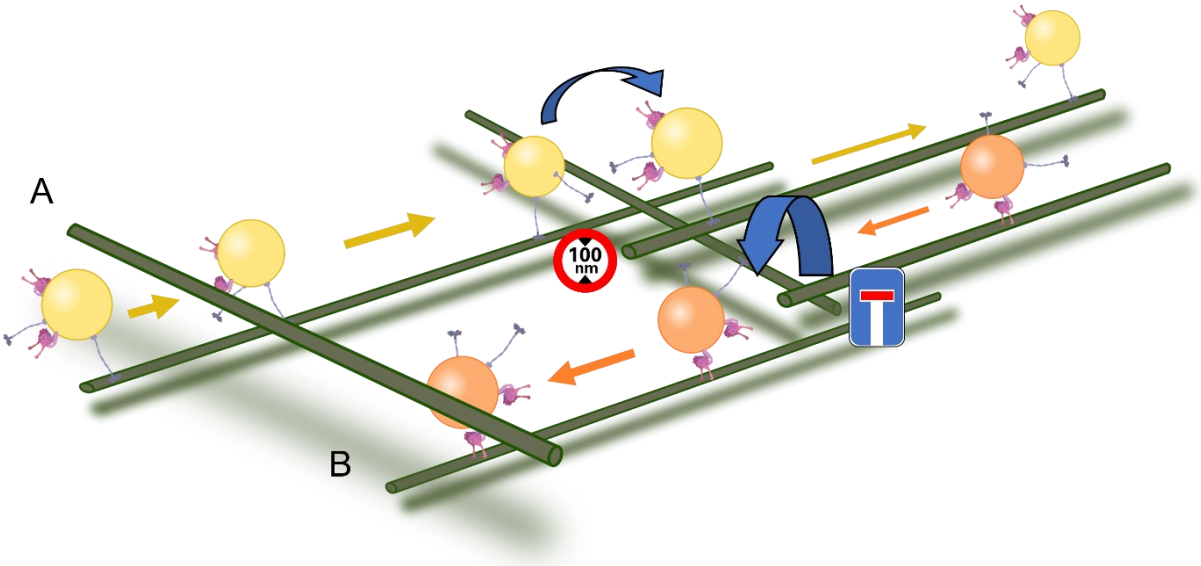

Figure 5 Cargo transport at cytoskeletal intersections and MT-ends. A Cargos that encounter intersecting MTs with an axial separation of less than ~100 nm face a major obstacle. Transport is slowed down; molecular motors are stalled until the obstacle can be overcome for instance by switching tracks. **B** Cargos encountering MT-ends must switch tracks to reach their destination as well. After short pause times of the motors at MT ends, possible remodeling of MTs, cargos switch tracks and proceed to their destination.

MT functionality with regard to dynamicity and stability is regulated by different factors such as MAPs, Ca^{2+}- as well as redox homeostasis. The MT organization, in terms of polymer lengths, number, the spatial distribution of individual MTs as well as the axial distribution of cytoskeletal intersections contributes to the nature and regulation of neuronal transport. Furthermore, the neuronal transport is orchestrated in an interplay with the other factors named above such as PTM, scaffolding proteins, or MAPs. The association of MT breakdown seen in neurodegeneration creates the desire to directly visualize the MT array to improve our understanding of actual events taking place. Furthermore, visualization of the MT array enables the opportunity to investigate direct outcomes of possible interventions. However, the small caliber of neuronal processes and densely packed MT organization, make the visualization and especially quantification of the MTs extremely challenging. Up to date the gold standard method to analyze axonal or dendritic MT organization is the very labor intense serial-section electron microscopy. Nevertheless, the development of nanobodies during the last decade and their application in fluorescence-based super-resolution microscopy techniques offers new possibilities to visualize neuronal MT structure and

organization (Mikhaylova et al., 2015). The application of computational strategies to analyze and quantify MT networks imaged by single-molecule localization microscopy (SMLM) paves the way to investigate neuronal MTs in axons and dendrites (Zhang et al., 2017) in response to regulating factors named above in an unseen extent.

The Microtubule-Associated Protein Tau

One of the first-ever observation of structures containing the tau protein, although in an aggregated and pathological state, was made by Alois Alzheimer in his 1907 publication "Eine eigenartige Erkrankung der Hirnrinde" (Alzheimer et al., 1995). Alzheimer visualized extracellular deposits in the cerebral cortex using an, at the time recently developed, silver staining method and described degenerated neurons with intracellular bundles of fibrils (Arendt et al., 2016). In the 1960s those fibrils, called neurofibrillary tangles (NFTs), were visualized in a high resolution by electron microscopy. Due to the appearance of two filaments as the principal constituent of the NFTs that seemed to be bound helically around each other, they were called paired helical filaments (PHFs) (Terry, 1963; Kidd, 1963). It took more than another 20 years of research until those filaments' structural and molecular nature was further elucidated. Through additional electron microscopy studies, it was revealed that PHFs are made up of double-helical stacks of C-shaped subunits (Wischik et al., 1985; Crowther and Wischik, 1985). Immunolabeling of PHFs showed, that the structural cores of those subunits were made up of a sequence from a protein that was purified already in the mid-70s (Anderton et al., 1982; Grundke-Iqbal et al., 1986). This heat-stable and highly soluble protein was isolated from porcine brain extracts as a tubulin-associated unit (tau) and identified as essential for MT assembly *in vitro* (Weingarten et al., 1975). As other proteins present in preparations of detergent-extracted cytoskeletons, and which are released from such preparations by MT-depolymerizing buffers, tau was assigned as a microtubule-associated protein (MAP) (Duerr and Pallas, 1981).

Tau is an abundant protein in the nervous system of vertebrates where it is enriched in the axonal compartment of nerve cells (Binder et al., 1985). However, tau is also found in the somatodendritic compartment of neurons as well as in astrocytes (Papasozomenos and Binder, 1987) and oligodendrocytes (Migheli et al., 1988; Klein et al., 2002). In the human central nervous system (CNS) six different isoforms of tau are expressed ranging from 352 to 441 amino acids. In the peripheral nervous system (PNS), an additional longer tau isoform, "big tau", is expressed having 254 additional amino acids (Goedert et al., 1992; Nunez and Fischer, 1997). Based on the biochemical properties of the protein, tau can be subdivided into four different regions: the N-terminal region (NTR), also known as the projection domain since it projects away from MTs when bound to them, the proline-rich region (PRR), the microtubule-binding region (MBR) and the carboxy-terminal region (CTR). The isoforms differ by the presence or absence of three alternatively spliced exons 2, 3, and 10, respectively. Exons 2 and 3 encode for two small inserts (N1 and N2) in the NTR, while exon 10 encodes for a repeat region (R2) within the MBR (Goedert et al., 1989) **(Figure 6).**

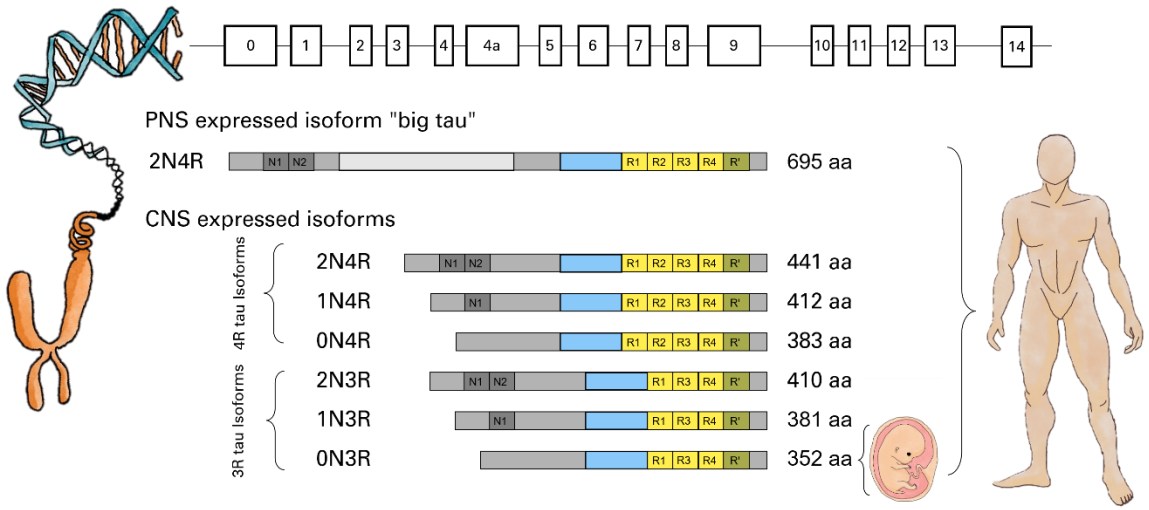

Figure 6 The MAPT gene, the PNS expressed isoform "big tau", and the six tau isoforms expressed in the CNS. Tau is encoded by a single gene located on chromosome 17q21.31 coding for 16 exons (white boxes). The exons E1, E4, E5, E7, E9, E11, E12, and E13 are constitutively spliced. Alternative mRNA splicing of E2, E3, and E10 gives rise to different isoforms. E0, which is part of the promoter, and E14 are noncoding. E6 and E8 are not transcribed in the human brain. E4a is expressed in the peripheral nervous system and gives rise to "big tau". In the fetal CNS, only the shortest tau isoform having 352 amino acids (aa) is expressed, while the remaining isoforms are additionally expressed in adult brains. The microtubule-binding region with the repeats (R1–R4) are depicted in yellow, with three isoforms having four repeats (4R) and three isoforms having three repeats (3R). The proline-rich region is shown in blue, the small inserts (N1 and N2) in the N-terminal domain (grey) are shown in dark grey, the pseudorepeat region (R') in the C-terminal domain (grey) is depicted in grey-yellow.

Tau belongs to the class of intrinsically disordered proteins (IDPs) and another possibility to distinguish the regions of tau is their level of disorder (Cleveland et al., 1977; Brandt et al., 2020). Proteins of that type are also known as hybrid proteins since they possess more structured regions like the MBR or CTR and intrinsically disordered regions (IDRs) such as the NTR and PRR (Uversky, 2015; Brandt et al., 2020). IDPs are amenable to post-translational modifications (PTMs) and known to interact with many different partners and serve thereby as hubs for cellular protein-interaction networks (Uversky, 2015). The NTR is known to be important for interactions with a variety of membrane-associated proteins (Brandt et al., 1995). Among them are annexins, which are membrane-binding proteins involved in the organization between the cytoplasm and cellular membranes (Gauthier-Kemper et al., 2018). Others are synaptic vesicle-associated proteins, such as Synapsin-1 or Synaptotagmin-1 which are involved in exocytosis and neuronal transmitter release. Furthermore, interactions of the NTR with signaling proteins of the 14-3-3 family are reported which are in turn involved in a broad spectrum of signaling pathways (Stefanoska et al., 2018; Chen et al., 2019). However, interactions with proteins of the 14-3-3 family are also reported for regions of the PRR and MBR of tau, indicating that these interactions are not a unique feature

of the NTR (Sadik et al., 2009b; Sadik et al., 2009a; Joo et al., 2015). Especially the PRR exhibits remarkable interactions with proteins involved in signaling pathways including kinases of the scr-family such as fyn (Lee et al., 1998; Klein et al., 2002) and protein phosphatase PP2A/Bα (Sontag et al., 2012). The PRR got its name due to the relatively high content of proline which is about 20% higher than the average occurrence of the amino acid in human proteins. Moreover, the PRR is the prime region for phosphorylation of tau (22 predicted sites) due to a high content of serine and an above-average amount of threonine (Brandt et al., 2020). The PRR is neighboring the MBR and the main interaction partner of tau within cells are microtubules while the vast majority of the protein (>90%) is bound to them (Konzack et al., 2007; Weissmann et al., 2009). However, this does not mean that the same tau molecules are bound to MTs at the same time. The interaction with MTs is a highly dynamic process with frequent de- and re-attachment of tau within a few milliseconds (Janning et al., 2014). The MT-binding is mainly fulfilled by its three or four repeat regions (R1-R4) in the MBR but there are also interactions with MTs mediated by flanking regions in the PRR and CTR (Brandt and Lee, 1993; Kadavath et al., 2015; Niewidok et al., 2016; McKibben and Rhoades, 2019). Within each imperfect repeat of 31 to 32 amino acids, stronger and weaker MT-interacting regions are present, while the strongly interacting residues of the repeats include the highly conserved SKI(C)GS motif (Kellogg et al., 2018; Brotzakis et al., 2021). In addition to MT-binding, there are several other interaction partners linked to the MBR including heat shock proteins (Taylor et al., 2018), actin (Correas et al., 1990), end binding proteins (Ramirez-Rios et al., 2016) or histone deacetylase 6 (HDAC6) (Ding et al., 2008). The variety of different proteins and cellular components tau interacts with appear to be different for the NTR, the PPR, and the MBR. Interestingly none of the interaction partners is specific for the CTR. Overall, tau is interacting with about 60 different interaction partners, while one-third of the interactions were reported to be sensitive to phosphorylation (Trushina et al., 2019). Tau is bearing about 85 phosphorylation sites (Hanger et al., 2007) and during tauopathies, such as Alzheimer's disease (AD), tau redistributes from the axonal to the somatodendritic compartment and exhibits pathologically increased phosphorylation (hyperphosphorylation) at numerous sites. The proportion of tau being phosphorylated at given sites is significantly higher in PHF-tau compared to biopsy-derived normal tau, meaning that pathological phosphorylation of PHF- or NFT-tau can be seen as a stoichiometric increase in the amount of phosphate at selected sites (Matsuo et al., 1994; Morishima-Kawashima et al., 1995; Eidenmüller et al., 2001) Hence, it is not surprising that phosphorylation is the best studied PTM of the tau protein so far. *In vitro,* phosphorylation of residues within the MBR repeats, especially of the universally conserved Ser262, drastically attenuates MT binding and reduces MT assembly

(Kellogg et al., 2018; Brotzakis et al., 2021). However, investigations of AD brains show only weak or partial phosphorylation of these residues when compared to healthy controls (Hasegawa et al., 1992; Neddens et al., 2018). To be noted, the idea that phosphorylation and dephosphorylation cycles are directly responsible for tau's rapid binding dynamics conflicts with the dwell time of individual molecules on the MTs. With about 25 possible interactions of a tau molecule with MTs per second, the catalytic rate of protein kinases (~10 reactions/s) is too slow to directly modulate tau-MT binding kinetics (Janning et al., 2014). The observed rapid "kiss and hop" behavior with MTs as well as tau's intrinsic disorder, and thus the high post-translational modifiability provides an explanation of how tau can interact with a large interactome in different cellular compartments. Apart from that, the highly dynamic interaction of tau with MTs gives an explanation for why axonal transport is not impeded by tau under physiological conditions (Yuan et al., 2008; Yuan et al., 2013) although tau and molecular motors compete for the same binding domains on MTs (Hagiwara et al., 1994) **(Figure 7)**. However, changes in PTMs that change tau's interaction with MTs or interactions with other binding partners might lead to transport deficits as described for tauopathies (Combs et al., 2019).

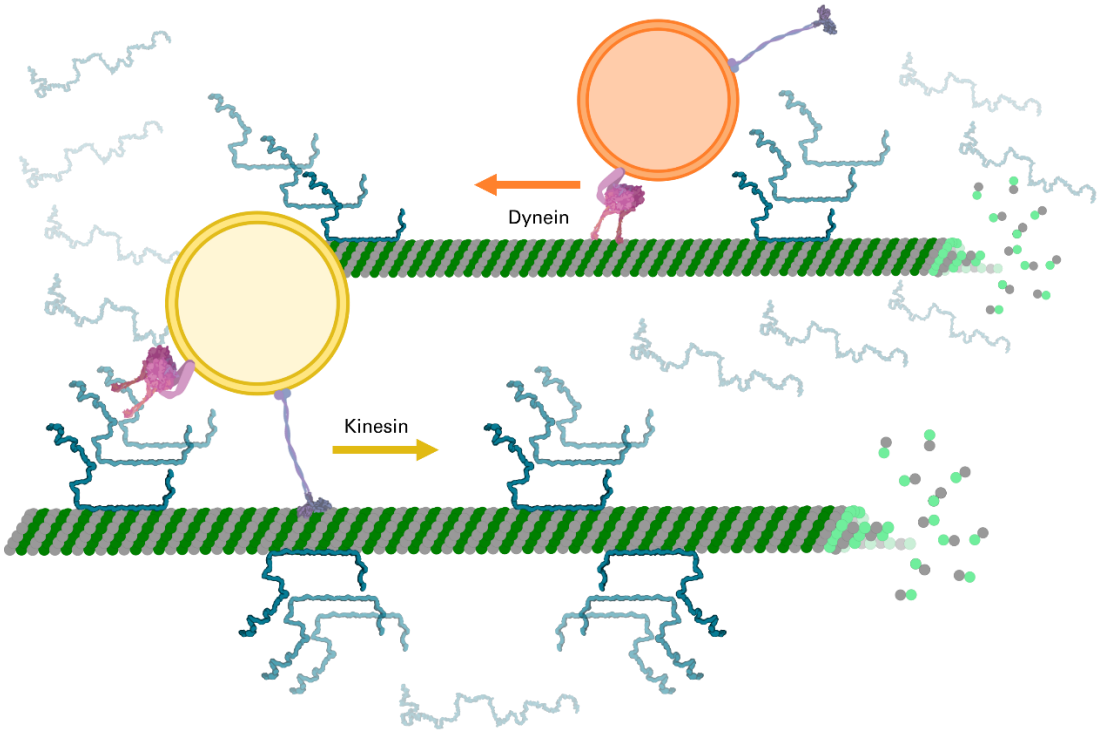

Figure 7 Kiss-and-Hopp interaction of Tau with MTs. The schematic shows dynamically interacting tau molecules (blue) with MTs as well as MT-dependent transport. After a short dwell time of several milliseconds (~40 ms in case of full-length tau), the MAP detaches from a MT, diffuses freely in the cytoplasm, and reattaches to the same MT, or binds to a neighboring one. The mean lateral distance between the two closest tau molecules bound to the same MT simultaneously is ~45 nm. Given that the average speed of kinesin or dynein is ~1 µm/s, the molecular motors can proceed ~40 nm during the mean dwell time of tau. Hence, molecular motors can readily proceed without being impeded by tau being in their way (Janning et al., 2014).

Anyhow, practically all phosphorylation sites linked to tauopathies are weakening the interaction of tau with MTs. Based on mass spectrometric analysis of PHF-tau derived from AD brains, 38 phosphorylated sites have been identified (Hanger et al., 2007). Out of those, 10 sites represent major phosphorylation sites and can be termed hyperphosphorylated (Morishima-Kawashima et al., 1995). Interestingly the vast majority of those sites, namely 34, and all of the 10 major phosphorylation sites are located in the PRR and CTR, flanking the MBR (Trushina et al., 2019). Experimental evidence suggests that phosphorylation at these major phosphorylation sites of tau results in structural changes and induces a "gain of toxic function" in neurons (Rapoport and Ferreira, 2000; Fath et al., 2002; Shahani et al., 2006). Furthermore, phosphorylation results in the accumulation of insoluble tau and induces neurodegeneration (Shahani et al., 2006). Thus, phosphorylation of tau is of great importance, and changes in the pattern, stoichiometry, and dynamics of phosphorylation can be linked to functional changes in physiological as well as pathological conditions (Trushina et al., 2019). As mentioned earlier, the PRR, as well as CTR, also directly contribute to tau's MT interaction. In the PRR, especially serine residues, such as the major phosphorylation site S235, perform interactions with parts of α- and β-tubulin while MT-binding of the CTR is primarily performed by lysine residues (Brotzakis et al., 2021). The CTR contains a segment that appears to be a truncated additional repeat region (Chapin and Bulinski, 1992). This pseudo-repeat region (R') in the C-terminus is highly conserved and remarkably enhances MT binding in axon-like processes independent of tau isoforms. Phosphomimicking or -blocking within the R'-region does not influence the tau-MT interaction. However, hyperphosphorylation at disease relevant positions at C-terminal sites flanking the MBR plus R' strongly attenuates the tau-MT binding (Niewidok et al., 2016).

Apart from phosphorylation, other PTMs gained interest in recent years. Especially acetylation of tau seems to be of relevance and is involved in the formation and maturation of NFTs (Min et al., 2010; Cohen et al., 2011; Irwin et al., 2012; Grinberg et al., 2013). Acetylation mimicking studies including residues K174, K274, and K280 induced tauopathy-like deficits such as neuronal loss, synaptic dysfunction, and cognitive impairment in mouse models (Min et al., 2015; Tracy et al., 2016). Acetylation of K280 within the so-called PHF-6* motif is present in several transgenic mouse models (Cohen et al., 2011; Iba et al., 2013; Song et al., 2015). Furthermore, it is found in tau lesions of human brain samples from all 4R-tauopathies supporting the pathological role of tau acetylation (Cohen et al., 2011; Irwin et al., 2012; Irwin et al., 2013; Grinberg et al., 2013). Patients with forms of frontotemporal dementia (FTD) exhibit a genetic deletion of lysine 280 (ΔK280) (Rizzu et al., 1999). Both deletion, as well as acetylation of lysine, lower the overall charge within the respective

region. To this day, the full complement of enzymes involved in tau acetylation is not well characterized. Tau has the capability to perform self-acetylation as it is known for auto-regulated acetyltransferases such as MYST-family acetyltransferases (Cohen et al., 2013). However, it could be shown that the acetyltransferases p300 and the CREB-binding protein (CBP) can acetylate tau (Min et al., 2010; Cohen et al., 2011; Cook et al., 2014a) and that sirtuin 1 deacetylase (SIRT1) and HDAC6 are major tau deacetylases (Cook et al., 2014b; Carlomagno et al., 2017). Acetylation and deacetylation of lysins and thereby restoring or neutralizing the positive charge seems to have residue-specific impacts. Tau deacetylation of residues within the PRR (K174) mediated by SIRT1 leads to a reduced pathogenic tau aggregation in mice (Min et al., 2018). CBP and the homologous p300 acetyltransferase were shown to be capable of acetylation of lysins of tau's KXGS motifs within the MBR (Min et al., 2010; Cohen et al., 2013; Cook et al., 2014a). The acetylation within the MBR appears to be important to prevent tau filament assembly. In accordance, deacetylation of the KXGS motifs seems to promote tau aggregation and is regulated by HDAC6 (Carlomagno et al., 2017). Interestingly, acetylation of the four lysine residues from the KXGS motif is found in tau of normal brains while it is reduced in tauopathy brains (Cook et al., 2014b). The expression levels of both deacetylases have been investigated in AD brains while lowered expression of SIRT1 (Julien et al., 2009) but increased levels of HDAC6 (Ding et al., 2008) were found, endorsing the opposing effects of both enzymes. In addition, acetylation and deacetylation of tau can affect its cellular localization. Under stress conditions, in accelerated aging models as well as in AD brains, Tau-K174 acetylation by CBP leads to the shuttling of tau in the nucleus. Once there, it causes changes in the transcription profile of cells increasing their translation capacity resulting in energy depletion. SIRT6, a lysine deacetylase restricted to the nuclear compartment, can counteract this by restricting the permanence of hyperacetylated tau in the nucleus (Portillo et al., 2021). In this context, it is noteworthy that PTMs and in this case, acetylation can of course affect tau and tau aggregation also indirectly. The heat shock protein 90 (HSP90), a known chaperone of tau, is kept inactive while acetylated. HDAC6 deacetylates HSP90 and catalyzes thereby its activation enhancing tau filament formation (Cook et al., 2012). Acetylation of tau is also of interest since crosstalk with phosphorylation events were reported (Kaluski et al., 2017; Carlomagno et al., 2017) but also because lysine residues are targets for other PTMs such as methylation, glycation, SUMOylation or ubiquitination (Yang and Seto, 2008). Methylation of tau is barely studied, and the functional effects are mostly unknown. Acetylation and ubiquitination of the same residues could be shown in mouse models and some sites were also target for lysine methylation (Morris et al., 2015). Furthermore, it could be shown *in vitro* that methylation of tau can attenuate the aggregation propensity (Funk et

al., 2014). Glycation, the addition of a carbohydrate to lysine, was shown to promote tau polymerization and stabilized tau aggregates *in vitro* but it does not induce tau aggregation *per se*. However, it might interfere with tau degradation (Necula and Kuret, 2004). The addition of a small ubiquitin-like modifier protein to lysine residues, called SUMOylation, seems to be triggered by phosphorylation of tau and was shown to inhibit ubiquitination as another PTM (Luo et al., 2014). Out of 44 lysine residues in full-length tau, there are 17 sites reported to be ubiquitinated (Morris et al., 2015; Munari et al., 2020). The main role of ubiquitination is the regulation of protein clearance by proteasomal or autophagy pathways. Thus interference with ubiquitination is suggestive of an impaired removal of tau and may lead to tau accumulation and aggregation (Chesser et al., 2013). In this regard, synaptic accumulation of hyperphosphorylated tau oligomers was observed in synaptosomes of AD brains having a compromised ubiquitin/proteasome system (Tai et al., 2012). However, ubiquitin is mainly found on PHF- and NFT-tau in brains of AD patients (Mori et al., 1987; Morishima-Kawashima et al., 1993) rather than on "pre-tangles" of tau (Bancher et al., 1991; Iwatsubo et al., 1992). This indicates that ubiquitin is primarily linked downstream to aggregated tau after the formation of inclusions as a mechanism of compensation for tau accumulation. On the other hand, in more recent studies a strong association was found between ubiquitination and a truncated variant of tau cleaved at aspartic acid 421 (Tau-C3) during the early maturation of NFTs (García-Sierra et al., 2012). Cleavage of tau, as another form of PTM, is found in transgenic mice as well as in the brains of AD patients. Several proteases have been shown to proteolyze tau, however, most of these enzymes do not appear to be principally accountable for tau clearance and promotion of abnormal tau removal. Instead, they seem to be responsible for the generation of modified tau species which contribute to tau pathology and may induce tau clearance in the aftermath (Chesser et al., 2013). Among these proteases is the calcium-activated protease calpain which generates a neurotoxic tau fragment (Park and Ferreira, 2005). Furthermore, activation of caspases, in particular, caspase-3 and -6 have been studied and were reported to be increased in neurons of AD brains (Su et al., 2001; Guo et al., 2004). While it is likely that caspases as markers for apoptosis also directly contribute to neurodegeneration, also non-apoptotic functions of caspases are known. (Kuranaga and Miura, 2007). However, a known effect of caspase-3 is the cleavage of tau at Asp421 creating the above-named Tau-C3 fragment. The exact functions, consequences, and pathological details of Tau-C3 are not fully understood but interestingly, the activation of caspases and generation of the TauC3 fragment were shown to be consistently preceding the formation of tangles in transgenic mice by hours or even days (Calignon et al., 2010). Accumulation of Tau-C3 was shown in animal models of traumatic brain injury (Glushakova et al., 2018) and exists in both soluble

and insoluble states in the brain of AD transgenic mice where it is associated with tangle formation and neurodegeneration (Zhang et al., 2009). Moreover, Tau-C3 was also associated with pretangles in neurons and dystrophic neurites of AD brain samples (Rissman et al., 2004). Caspase-cleaved tau was also proven to be present in brains of patients with progressive supranuclear palsy (PSP) and increased levels of Tau-C3 were observed in brains of patients with AD and FTD (Zhao et al., 2015). Furthermore, Tau-C3 has pro-apoptotic effects suggesting a vicious cycle for its own generation (Chung et al., 2001) and induces toxicity in neuronal cultures (Matthews-Roberson et al., 2008; Quintanilla et al., 2009). How Tau-C3 exerts its neurotoxic functions remains unknown and especially the early occurrence within tau pathology underlines the need for a better understanding of the molecular and cellular events.

Historically PHFs and NFTs were considered to be the toxic entities in AD and other tauopathies. Tau pathology in form of NFTs correlates with the severity of dementia and the regional distribution of NFTs is apparently associated with the progression of AD (Braak and Braak, 1991; Braak and Braak, 1995). However, over the last decade more and more evidence accumulated that pathologically modified monomeric and soluble oligomeric forms of tau should be considered as harmful tau species (Patterson et al., 2011; Flach et al., 2012; Lasagna-Reeves et al., 2012; Ward et al., 2012; Shafiei et al., 2017). Thus, there is a growing interest in finding ways to prevent the occurrence of aberrant tau species, defining pathways contributing to neurotoxicity, triggering the induction of their clearance, or their modulation rendering them harmless.

Modulating Strategies

Changes in the organization of MTs and MT regulating factors are common features of aging and neurodegenerative diseases like tauopathies. Hence, modulating MT dynamics and restoring or preserving neuronal MT stability can be a strategy to counteract the breakdown of the MT system and depending cellular processes. One possibility is to modulate MTs by addressing regulating factors such as MAPs. Another strategy is the direct interference of MTs using MT-targeting agents (MTAs).

The bottleneck for neuronal MT modulation, at least in case of the CNS, is that respective compounds must cross the blood-brain barrier. Promising brain-penetrant compounds are epothilones (Altmann et al., 2000). Epothilones bind to the β-tubulin subunit and induce tubulin polymerization similar to paclitaxel. One of the most prominent members of the epothilone family is Epothilone D (EpoD). The compound can reduce transport deficits in transgenic mouse models, enhances cognition by stabilizing axonal MTs, improves tau pathology, and is capable of reversing Aβ-induced spine loss in mouse models (Brunden et al., 2010; Barten et al., 2012; Zhang et al., 2012; Penazzi et al., 2016). In addition, EpoD exhibits desirable pharmacokinetic and pharmacodynamic properties, such as a longer brain than plasma half-life, prolonged accumulation within the CNS, and the ability to elicit delayed increase in long-lived MTs at very low concentrations (Brunden et al., 2011). However, despite beneficial effects at early stages of neurodegenerative disease, accelerated disease progression has been shown in transgenic mice. Furthermore, neurotoxic effects on developing neurons in a dose-dependent manner have been identified (Clark et al., 2018; Clark et al., 2020). EpoD was investigated in a phase I clinical trial to evaluate safety, efficiency, and tolerability in patients with AD. Although the study was completed, further clinical studies were discontinued due to the lack of a mechanistic selectivity for neurons (Soliman et al., 2022). Another brain-penetrant MTA is the synthetic taxane-derivate TPI-287. Similar to EpoD, TPI-287 accumulates in the brain and exhibits the capability to promote MT polymerization likewise to paclitaxel (Fitzgerald et al., 2012). Preclinical studies with respect to its efficacy in models of neurodegenerative diseases were not published. However, the compound was investigated in two clinical trials in patients with AD and 4R-tauopathies, respectively. Recently it was reported that the conducted studies do not support the continued development of TPI-287 for tauopathies (Tsai et al., 2020). The attempt of direct action on MTs seems to be problematic most likely due to the critical importance of MTs in all cell types for various biological processes. Hence, an indirect modulation of MT organization by addressing regulating factors of neuronal MTs, such as the tau protein, might be a more promising approach.

One strategy is targeting the PTMs of tau and thereby modulating the MT system and tau pathology. As pathological phosphorylation at selected sites is a hallmark of tauopathies, targeting kinases like GSK3-β seems to be an obvious approach (Bakota and Brandt, 2016). The small molecule Tideglusib, an irreversible GSK-3β inhibitor, showed the ability to reduce tau phosphorylation, Aβ deposition, and neuronal loss in preclinical studies (Serenó et al., 2009) but failed in a clinical phase II trial to slow down cognitive or functional impairment in AD (Lovestone et al., 2015). Other compounds have been investigated targeting phosphorylation directly or indirectly, such as Dvaunetile or LY3372689. However, as with targeting the MTs directly, modifying PTMs of tau or other proteins might be problematic in general. Given that PTMs, like phosphorylation for instance, are very general modifications affecting and contributing to various protein functions and signaling pathways, interference is likely to cause unwanted side effects.

Although tau is a cytoplasmatic protein, it is also found in the interstitial fluid (ISF) and cerebrospinal fluid (CSF) and in absence of neurodegeneration. However, in the case of tauopathies, extracellular tau species are found at significantly higher levels in the ISF of the brain, and can pass into CSF (Yamada et al., 2011). Immunotherapeutic approaches targeting extracellular soluble or aggregated tau variants are thought to reduce the spreading of tau pathology between anatomically connected brain regions. Some antibodies have been shown to be taken up by neurons (Congdon et al., 2013; Collin et al., 2014), which is an important feature since tau levels within the cells are considered to be several orders of magnitude higher than in the ISF and CSF (Han et al., 2017). Hence, different anti-tau immunotherapeutic approaches have been assessed in clinical trials or are still under investigation. Among them are active immunization therapeutics using small peptides that mimic tau pathology epitopes, such as AAdvac or ACI-35. Furthermore, passive immunization strategies, employing monoclonal antibodies against tau are currently examined. These include antibodies against the N-terminal domain of tau, such as Gosuranemab, Tilavonemab, Semorinemab, and Zagotenemab. Other monoclonal antibodies are directed against the mid-domain of tau, such as BIIB076, or against potentially neurotoxic tau species like RG7345 (Vaz and Silvestre, 2020; Soliman et al., 2022). Another approach to reduce the amount of tau in cells are small molecules. The antisense oligonucleotide IONIS MAPTRx (BIIB080) inhibits the mRNA translation of tau. Preclinical data showed that the antisense oligonucleotide leads to a reduction in NFT spread and decreased neuronal loss in transgenic mice (DeVos et al., 2017). The compound is currently in a clinical phase I study to evaluate safety, tolerability, pharmacokinetics, and pharmacodynamics of IONIS-MAPTRx in patients with mild AD (Cummings et al., 2021).

Given that tau not only plays a role in regulating MT dynamics but is also interacting with numerous binding partners and contributes to signaling mechanisms, lowering total tau amounts might pose a risk as well. However, reduction of tau is a promising approach as tau shows a toxic gain of function in neurodegenerative diseases. This toxic gain of function involves the transition from a soluble intrinsically disordered monomeric protein to an aggregated and finally insoluble structured filament (Goedert, 2016). Thus, several small molecules have been developed aiming a reduction of tau aggregation and thereby preventing the toxic tau variants. Methylthioninium chloride (MTC) (commonly known as "methylene blue") was reported to inhibit tau aggregation *in vitro* (Wischik et al., 1996). Derivates of MTC have been developed and assessed in clinical trials. TRx0273, a reduced derivate of methylene blue (Baddeley et al., 2015), is currently investigated in a phase III clinical trial that should be completed by end of 2022. Another small molecule inhibitor of tau aggregation is ACI-3024. No preclinical data has been published, but according to data presented at conferences the compound disrupts tau aggregates in cell-based assays at nanomolar concentrations, prevents microglia activation and neuronal death induced by tau aggregates in primary brain cultures as well as in mouse models of tauopathy (Alzforum Therapeutics, 2021). The compound has been assessed in clinical trials and investigations are ongoing.

Recent findings acknowledge the view that soluble oligomeric tau species play an important role in tauopathies, convey neurotoxicity within cells and when secreted (Gerson et al., 2016; Shafiei et al., 2017; Marcatti et al., 2022). On the other hand, early oligomeric tau variants seem to lack seeding competence *per se* and oligomeric tau toxicity can be reversed in transgenic mice (Wegmann et al., 2016; Martinisi et al., 2021). The seeding capacity of oligomeric tau appears to require certain PTMs, such as increased phosphorylation at specific sites or proteolytically C-terminal cleavage (Ercan-Herbst et al., 2019; Wesseling et al., 2020). Those PTMs, in turn, provoke the generation of soluble tau oligomers and drive their secretion (Plouffe et al., 2012; Ozcelik et al., 2016). Hence, early targeting of oligomeric soluble tau variants by inhibiting their aggregation or inducing their clearance in combination with a moderate reduction of the overall tau amount might prevent the progression of tauopathies and counteract the associated MT breakdown. This could be accompanied by targeting other components of the nervous MT system, such as elevation of MAP6 levels and thereby stabilize MTs compartment-specific in the axon of neurons.

OBJECTIVES

"It is very easy to answer many of these fundamental biological questions; you just look at the thing!"

(Richard Feynman, 1959)

Aim of this thesis is to investigate how the neuronal MT array, its dynamics, and MT-dependent transport are affected by cytoskeletal stabilization and modifications of the microtubule-associated protein tau. Strategies to modulate the MT-organization and dynamics, tau-MT interaction and MT-dependent transport behavior should be explored, and investigations of tau-aggregation inhibitors presented.

Effects on the organization of MTs are studied by localization-based super resolution microscopy using DNA-PAINT (Point Accumulation for Imaging in Nanoscale Topography) and algorithm-based reconstruction of the MT-cytoskeleton. MT- and tau dynamics are examined by live-cell imaging and the utilization of a Fluorescence Decay after Photoactivation (FDAP) approach. Quantification of MT and tau dynamics are performed by application of reaction-diffusion models. Downstream effects on MT-dependent transport are studied by spinning disk confocal microscopy and algorithm-based tracking of vesicle movement in model neurons.

RESULTS

Caspase-cleaved tau is senescence-associated and induces a toxic gain of function by putting a brake on axonal transport

Conze, C., M. Rierola, N. I. Trushina, M. Peters, D. Janning, M. Holzer, J. J. Heinisch, T. Arendt, L. Bakota, and R. Brandt. 2022. *Molecular Psychiatry.* doi:10.1038/s41380-022-01538-2.

Super-resolution imaging and quantitative analysis of microtubule arrays in model neurons show that epothilone D increases the density but decreases the length and straightness of microtubules in axon-like processes

Conze*, C., N. I. Trushina*, M. Holtmannspötter, M. Rierola, S. Attanasio, L. Bakota, J. Piehler, and R. Brandt. 2022. *Brain Research Bulletin.* doi:10.1016/j.brainresbull.2022.10.008

(* Shared first authorship)

Tau and α-synuclein shape microtubule organization and microtubule-dependent transport in neuronal dendrites

Rierola, M., N. I. Trushina, N. Monteiro-Abreu, C. Conze, M. Holtmannspötter, R. Kurre, M. Holzer, T. Arendt, J. J. Heinisch, R. Brandt, and L. Bakota. 2022. *BioRxiv*. doi:10.1101/2022.06.09.495530.

Quantitative live cell imaging of a tauopathy model enables the identification of a polypharmacological drug candidate that restores physiological microtubule regulation

Pinzi*, L., C. Conze*, N. Bisi, G. Dalla Torre, N. Monteiro-Abreu, N. I. Trushina, A. Soliman, A. Krusenbaum, M. Khodaei Dolouei, A. Hellwig, M. S. Christodoulou, D. Passarella, L. Bakota, G. Rastelli#, and R. Brandt#. 2022. *BioRxiv*. doi:10.1101/2022.10.31.514565

(* shared first authorship)

(# shared corresponding authorship)

CONCLUSIONS

Scope of this thesis was to study how the neuronal MTs, their dynamics, MT-dependent transport and tau-MT interaction are affected by MT stabilization or modifications of the microtubule-associated protein tau. Furthermore, the thesis aimed to investigate inhibitors of tau aggregation.

In the first study presented (Conze et al., 2022a) a particular PTM of tau, the caspase-3 cleavage of full-length tau at Asp421 creating a truncated proteoform called Tau-C3, was investigated. We showed that Tau-C3 is increased in the hippocampus of senescent mice and is also present in AD post-mortem human brain samples. To elucidate how Tau-C3 affects neuronal function and exerts potentially neurotoxic effects, the dynamic interaction with MTs using FDAP and single molecule tracking (SMT) of Tau-C3 was explored. The experiments showed that Tau-C3 has a drastically reduced dynamic interaction with MTs and dwells longer on individual MTs in axon-like processes of model-neurons as well as in axons of primary neurons. One of tau's role in the axon is to regulate MT dynamics (Qiang et al., 2018). Hence, we investigated the influence of a longer dwelling tau proteoform on tubulin dynamics by FDAP, finding that MT dynamics are unchanged in the presence of caspase-3 cleaved tau. However, as Janning et al. (2014) proposed that longer residence times of tau molecules on MTs may impair transport, we investigated the effect of Tau-C3 on axonal transport. Indeed, mitochondrial as well as vesicle transport are slowed down in Tau-C3 expressing cells. Analyzing individual vesicle trajectories revealed that the frequency of stalled transport and changes in directionality of individual vesicles is increased in presence of Tau-C3, indicating that less dynamic tau molecules act as temporary roadblocks on MT tracks. To further investigate the effect of Tau-C3, we analyzed its effect on CA1 pyramidal neurons, who's dendritic arbor is strongly reduced in AD brains (Flood, 1991). Tau-C3 leads to compartment specific dendritic simplification in the distal parts of the apical dendritic tree suggesting that consequences of an impaired transport occur first in regions which are most remote from the cell body. As tau is enriched on the dynamic regions of MTs (Black et al., 1996) we tested whether a stabilization of MTs using EpoD can modulate the dynamic interaction of tau with MTs. We observed that a low nanomolar treatment with 5nM EpoD restored the dynamic interaction of Tau-C3 similar to levels of full-length tau in presence of the MT stabilizer. In consequence, we also observed a return of vesicle transport parameters comparable to full-length tau under those conditions. However, in presence of EpoD and overexpression of human tau, an impaired transport of vesicles was observed. Notwithstanding the negative effect of EpoD on axonal transport in presence of both

exogenous tau constructs, the study presents a new type of toxic gain of function of tau and introduces microtubule-targeting drugs as pharmacological modifiers of MAP-microtubule interaction, thereby restoring the interaction of tau with MTs.

In accordance with the results presented in Conze et al. (2022a), a recent study showed, that low nanomolar concentrations of 10nM EpoD are sufficient to perturb axonal transport parameters at physiological tau levels (Clark et al., 2020). Hence, we were interested in defining the structural and organizational changes that axonal MTs undergo after treatment with EpoD presented in the second study of this thesis (Conze et al., 2022b). We have addressed this task by implementing super-resolution imaging and quantitative analysis of neuronal MTs employing three-dimensional DNA-PAINT (Point Accumulation for Imaging in Nanoscale Topography). The single-molecule localization microscopy (SMLM) technique and algorithm-based quantification of MT organization revealed that MT length distribution was shifted to shorter ones, while the MT density in axon-like processes of model neurons was increased. The MT-filament straightness was significantly decreased after EpoD treatment and the amount of MT filaments crossing each other in a proximity of less than 150 nm was increased. Given that Bálint et al. (2013) showed that a separation of intersecting MT tracks in close proximity represents a major obstacle for transport as well as the findings of Yogev et al. (2016) who reported that molecular motors pause when encountering microtubule-termini (**Figure 5**), our study provides an explanation for the transport deficits in presence of EpoD seen by Clark et al. (2020). Furthermore, the shift in the microtubule array properties after MT stabilization might explain the negative effect of EpoD on axonal transport reported in Conze et al. (2022a).

Tau is enriched in the distal region of axons and interacts especially with labile domains of MTs (Black et al., 1994; Black et al., 1996; Qiang et al., 2018), but it also has dendritic functions (Ittner et al., 2010). Hence, we used the super-resolution imaging approach presented in Conze et al. (2022b) to investigate the effect of tau on MTs in the dendritic compartment presented in the third study of this thesis (Rierola et al., 2022). The general aim of the study was to elucidate how physiological tau levels affect the morphology of dendrites by comparison of hippocampal pyramidal neurons from wildtype and tau knockout mice. The absence of tau lead to a more complex dendritic arbor in CA1 pyramidal neurons. Employing FDAP measurements of tubulin did not reveal any changes in the association and dissociation rate constants of tubulin nor in the estimated amount of polymerized tubulin in the primary neurons. However, DNA-PAINT based super-resolution imaging and quantification of the organizational properties of the dendritic MT array revealed, that in absence of tau individual MTs are longer, straighter, and less intertwined

shown by a decrease in the density of intersecting MT filaments. Dendritic transport in hippocampal tissue cultures was analyzed using high-speed volumetric lattice light-sheet microscopy. By utilizing the principles of evaluating transport introduced in Conze et al. (2022a), an increased processivity of transport was seen. While pause times of vesicles were unchanged, a significant decrease in directional changes was observed. If one considers the structural changes of MT tracks shown by the DNA-PAINT SMLM approach, this transport behavior is again in agreement with the findings of Bálint et al. (2013) and Yogev et al. (2016). The results suggest that the depletion of tau has a positive effect on the efficiency of dendritic transport facilitating the development of a more complex dendritic arbor in CA1 pyramidal neurons. Furthermore, analysis of the full protein profile of tau knockout hippocampal tissues showed that in absence of tau, proteins from the kinesin and dynein families were elevated and that α-synuclein, a classical presynaptic protein, is highly overexpressed. Analysis of transport in control samples indicated that elevated levels of α-synuclein are responsible for the increase in processivity of transport observed in neurons from tau knockout mice. Moreover, it was shown that tau and α-synuclein positively correlate in young mice but negatively correlate in old animals, while analysis of post-mortem tissue of AD patients revealed that pathological aging induces a positive correlation between both proteins. Hence, the study showed that physiological tau modulates the dendritic morphology and MT based transport by its effect on the structural organization of the dendritic MT array and suggests that this process can also be driven by α-synuclein.

The investigation of tau's physiological function provides important insights to our understanding of tau pathology and neurodegeneration (Arendt and Bullmann, 2013). Nevertheless, abnormal forms of tau have been shown to be toxic and contribute to pathological conditions (Arendt et al., 2016; Conze et al., 2022a). Scope of the fourth study (Pinzi et al., 2022) was therefore the implementation of a cell-based approach allowing the investigation of a pathological tau-MT interaction and the identification of compounds restoring physiological tau-MT interaction. The deletion of Lys-280 from full-length tau leads to an aggregation-prone tau variant, TauΔK280 (Rizzu et al., 1999). We used TauΔK280 expression to implement a cellular assay suitable for screening of a panel of compounds with predicted tau and kinase modulating activity. We employed FDAP experiments in model neurons as well as primary neuron cultures (DRG neurons) to demonstrate that TauΔK280 exhibits an increased effective diffusion constant corresponding to a ~10% reduced binding to MTs compared to wild-type tau. Based on the aggregation-propensity of the TauΔK280 construct, that was demonstrated in cell-free assays, the reduction of MT-binding was likely to be caused by the formation of small soluble tau oligomers. This was

further supported as the prolonged expression TauΔK280 in DRGs resulted in the formation of higher aggregates, which were positive for amyloid staining. Hence, we demonstrated that the approach provides a tool to identify compounds modulating tau oligomerization and tau-MT interaction in living neurons. We tested 2-phenyloxazole (PHOX) derivatives with predicted tau anti-aggregation activity at subtoxic concentrations, validated by combined cytotoxicity and metabolic assays, with our live-cell imaging approach. The screening of 12 compounds lead to the identification of PHOX15 being able to reduce the effective diffusion constant close to the level of the wild-type tau protein in model-neurons as well as primary neuron cultures. The determined IC_{50} value of the compound restoring the tau-MT interaction was in the concentration range of the expressed tau construct. Taken together the findings suggest that the identified compound counteracts tau oligomerization and restores physiological tau-MT interaction. Cell-free aggregation assays showed that PHOX15 can reduce heparin-induced filament formation by about ~20% while it failed to dissolve preformed aggregates from human brain samples. Hence, our results indicate that PHOX15 inhibits the first phase of tau aggregation by interfering with the oligomerization of the protein. Proteomic and phosphoproteomic analysis of PHOX15-exposed neural cells showed that none of the more than 60 interaction partners of tau were up or downregulated. This is suggesting that the changes in MT interaction and aggregation are independent of the tau interactome. Given that increased phosphorylation and aggregation of tau are hallmarks of tauopathies, it was an interesting finding that chemoinformatic analysis predicted a polypharmacological activity of PHOX15 towards glycogen synthase kinase 3β (GSK3β) and cyclin dependent kinase 5 (Cdk5) which was confirmed by experimental *in vitro* data. Kinase enrichment analysis from PHOX15-treated neurons reveled that the compound affected GSK3β-dependent phosphorylation in neural cells. The phosphoproteomics data showed that the phosphoprofile of endogenous tau was greatly reduced in the PRR of tau including major phosphorylation sites identified in PHFs from AD patients which are predicted to be phosphorylated by GSK3β and Cdk5. By employing a phosphoblocking tau construct, we investigated whether PHOX15 modulates tau-MT interaction by reducing tau phosphorylation at disease-relevant sites using our FDAP-approach. The compound increased tau-MT binding of wildtype tau but not of the phosphoblocking tau construct, indicating that PHOX15 increased tau MT-interaction by lowering phosphorylation at disease-relevant sites. The presented study introduces cell-based assays suitable for mechanism-based drug screening of full-length tau proteins with disease-relevant mutations and modifications. The investigation of tau oligomerization in combination with phosphorylation and tau MT-interaction allowed the identification of polypharmacological drug candidates with the potential to counteract tau-induced neurodegeneration.

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LIST OF PUBLICATIONS WITHIN THIS THESIS

Conze, C., M. Rierola, N. I. Trushina, M. Peters, D. Janning, M. Holzer, J. J. Heinisch, T. Arendt, L. Bakota, and R. Brandt. 2022. **Caspase-cleaved tau is senescence-associated and induces a toxic gain of function by putting a brake on axonal transport.** *Molecular Psychiatry*. doi:10.1038/s41380-022-01538-2.

Personal contribution to:

- Conceptualization of the study, design of all figures, writing of figure legends and material and methods of the study, reviewing the manuscript
- Cell culture, transfections, treatments of model-neurons
- Preparation of immunoblots from cell-lysates and their analytics
- Live-cell imaging of photoactivation experiments and analysis of tau and tubulin dynamics using reaction diffusion models
- Live-cell imaging of vesicle transport experiments, image deconvolution, tracking and analysis of transport parameters (implementing transport data analysis strategies at the lab)
- Statistical analysis of all data within the study, including supplementary material

Conze*, C., N. I. Trushina*, M. Holtmannspötter, M. Rierola, S. Attanasio, L. Bakota, J. Piehler, and R. Brandt. 2022. **Super-resolution imaging and quantitative analysis of microtubule arrays in model neurons show that epothilone D increases the density but decreases the length and straightness of microtubules in axon-like processes.** *Brain Research Bulletin.* doi:10.1016/j.brainresbull.2022.10.008

* Shared first authorship.

Personal contribution to:

- Conceptualization of the study, writing of the original manuscript draft including design of figures, figure legends as well as material and methods, reviewing the manuscript
- Cell culture, transfections, treatments, fixation, immunostaining of model neurons and primary hippocampal neuron cultures
- Super resolution imaging with DNA-PAINT using TIRF microscopy in HiLo mode, post-processing of image raw data, analysis and quantification cytoskeletal parameters (implementation of cytoskeletal SMLM-data analysis strategy at the lab)
- Statistical analysis of all data within the study

Rierola, M., N. I. Trushina, N. Monteiro-Abreu, C. Conze, M. Holtmannspötter, R. Kurre, M. Holzer, T. Arendt, J. J. Heinisch, R. Brandt, and L. Bakota. 2022. **Tau and α-synuclein shape microtubule organization and microtubule-dependent transport in neuronal dendrites.** *BioRxiv*. doi:10.1101/2022.06.09.495530.

Personal contribution to:

- Design of Figure 2 D and E, writing of corresponding figure legend as well as material and method section, reviewing the manuscript
- Analysis and quantification of DNA-PAINT derived super-resolution image data and quantification of cytoskeletal parameters

Pinzi*, L., C. Conze*, N. Bisi, G. Dalla Torre, N. Monteiro-Abreu, N. I. Trushina, A. Soliman, A. Krusenbaum, M. Khodaei Dolouei, A. Hellwig, M. S. Christodoulou, D. Passarella, L. Bakota, G. Rastelli#, and R. Brandt#. 2022. **Quantitative live cell imaging of a tauopathy model enables the identification of a polypharmacological drug candidate that restores physiological microtubule regulation.** *BioRxiv*. doi: 10.1101/2022.10.31.514565.

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Shared corresponding authorship.

Personal contribution to:

- Conceptualization of the study, design of figures 1, 2 and 3 as well as S3, writing of corresponding figure legends and material and method sections, reviewing the manuscript
- Creation of the aggregation prone tau construct (TauΔK280) using site-directed mutagenesis
- Cell culture, transfections, infections, and treatments of model neurons and primary DRG neurons (implementation of DRG neuron preparation at the lab)
- Live-cell imaging of photoactivation experiments and analysis of tau dynamics in model neurons and DRG primary neurons using a reaction diffusion model, screening of the PHOX compound set for tau aggregation inhibitors and dose-response assessment by FDAP experiments
- Assay implementation and conduction of combined toxicity and metabolic activity profiles for the PHOX compound set
- Statistical analysis of all data shown within figures 1,2, and 3 as well as for supplementary material figure S3

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AIS	axon initial segment
APP	amyloid precursor protein
ATP	adenosine triphosphate
CAMSAP	calmodulin-regulated spectrin associated protein
CBP	CREB-binding protein
CNS	Central nervous system
CSF	cerebrospinal fluid
CTR	carboxy-terminal region
DNA-PAINT	DNA point accumulation for imaging in nanoscale topography
EB	end binding protein
EpoD	Epothilone D
ER	endoplasmic reticulum
FDAP	fluorescence decay after photoactivation
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HDAC	histone deacetylase
IDP	intrinsically disordered protein
IDR	intrinsically disordered region
ISF	interstitial fluid
JIP1	JNK-interacting protein 1

KIF	kinesin superfamily protein
MAP	microtubule associated protein
MBR	microtubule-binding region
MT	microtubule
MTA	microtubule-targeting agent
MTC	methylthioninium chloride
NFT	neurofibrillary tangle
NTR	N-terminal region
PHF	paired helical filament
PNS	peripheral nervous system
PRR	proline-rich region
PTM	post-translational modification
R (R1-R4, R')	repeat region, pseudorepeat region
ROS	reactive oxygen species
SIRT	Sirtuin deacetylase
SMLM	single-molecule localization microscopy
-TIP or minus-TIP	minus-end tracking protein
+TIP or plus-TIP	plus-end tracking protein
TRAK	trafficking kinesin protein

CURRICULUM VITAE

Name:	Christian Conze, formerly Christian Gach
Address:	Delmestraße 70, 28199 Bremen, Germany
Phone:	0176/56819354
E-mail:	c_conze@outlook.de
Date of birth:	1st June 1986 in Aachen, Germany
Nationality:	German

PROFESSIONAL EXPERIENCE

10/2016 –05/2022	**Research Associate**, University of Osnabrück
09/2015 – 8/2016	**Research Associate / Student and Research Assistant**, Fraunhofer Institute for Molecular Biology and Applied Ecology (IME) ScreeningPort Hamburg
10/2011 – 09/2015	**Biological-Technical Assistant (BTA) / Student and Research Assistant**, Fraunhofer Institute for Laser Technology (ILT) Aachen

EDUCATION

10/2016 – present	**Doctoral Candidate**, Osnabrück University Doctoral Thesis at the **Department of Neurobiology**, Prof. Dr. Roland Brandt *Imaging of Tau and Microtubules to Study Mechanisms of Tau Pathologies and Neurodegeneration*
09/2014 – 05/2016	**Studies of Biotechnology**, FH Aachen University of Applied Sciences Master of Science Thesis at the **Fraunhofer Institute for Molecular Biology and Applied Ecology IME ScreeningPort Hamburg** *Development of a cell migration assay to analyze modulators of PHACTR1-dependent cell motility*
09/2008 – 08/2013	**Studies of Biotechnology**, FH Aachen University of Applied Sciences Bachelor of Science Thesis at the **Fraunhofer Institute for Laser Technology ILT Aachen** *The targeted single-cell transfer of 3T3 and K562 cells using Laser Induced Forward Transfer (LIFT)*
08/2006 – 07/2008	**Apprenticeship as Biological-technical assistant (BTA)**, Rheinische Akademie Köln gGmbH
06/2006	**Abitur**, Einhard Gymnasium Aachen

DECLARATION

I hereby declare that I have written the presented dissertation thesis “Imaging of Tau and Microtubules to Study Mechanisms of Tau Pathologies and Neurodegeneration” without unauthorized help from third parties and without using other than the specified references in the text. Nobody has directly or indirectly received pecuniary benefits for work done in connection with the content of the submitted dissertation. Concepts and or data directly or indirectly used in the thesis are clearly attributed to the references which they have been adopted from.

This dissertation thesis was not submitted in the same or a similar form, either domestically or abroad to any examining authority.

Osnabrück, 07.10.2022 Christian Conze

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